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On the roles of colour and scent in a specialized floral mimicry system.

Vereecken, N J ; Schiestl, F P

Abstract: BACKGROUND AND AIMS: Sexually deceptive orchids achieve cross-pollination by mimicking the mating signals of female insects, generally hymenopterans. This pollination mechanism is often highly specific as it is based primarily on the mimicry of mating signals, especially the female sex pheromones of the targeted pollinator. Like many deceptive orchids, the Mediterranean species *Ophrys arachnitiformis* shows high levels of floral trait variation, especially in the colour of the perianth, which is either green or white/pinkish within populations. The adaptive significance of perianth colour polymorphism and its influence on pollinator visitation rates in sexually deceptive orchids remain obscure. METHODS: The relative importance of floral scent versus perianth colour in pollinator attraction in this orchid pollinator mimicry system was evaluated by performing floral scent analyses by gas chromatography-mass spectrometry (GC-MS) and behavioural bioassays with the pollinators under natural conditions were performed. KEY RESULTS: The relative and absolute amounts of behaviourally active compounds are identical in the two colour morphs of *O. arachnitiformis*. Neither presence/absence nor the colour of the perianth (green versus white) influence attractiveness of the flowers to *Colletes cunicularius* males, the main pollinator of *O. arachnitiformis*. CONCLUSION: Chemical signals alone can mediate the interactions in highly specialized mimicry systems. Floral colour polymorphism in *O. arachnitiformis* is not subjected to selection imposed by *C. cunicularius* males, and an interplay between different non-adaptive processes may be responsible for the maintenance of floral colour polymorphism both within and among populations.

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On the roles of colour and scent in a specialized floral mimicry system

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• **Background and Aims** Sexually deceptive orchids achieve cross-pollination by mimicking the mating signals of female insects, generally hymenopterans. This pollination mechanism is often highly specific as it is based primarily on the mimicry of mating signals, especially the female sex pheromones of the targeted pollinator. Like many deceptive orchids, the Mediterranean species *Ophrys arachnitiformis* shows high levels of floral trait variation, especially in the colour of the perianth, which is either green or white/pinkish within populations. The adaptive significance of perianth colour polymorphism and its influence on pollinator visitation rates in sexually deceptive orchids remain obscure.

• **Methods** The relative importance of floral scent versus perianth colour in pollinator attraction in this orchid pollinator mimicry system was evaluated by performing floral scent analyses by gas chromatography-mass spectrometry (GC-MS) and behavioural bioassays with the pollinators under natural conditions were performed.

• **Key Results** The relative and absolute amounts of behaviourally active compounds are identical in the two colour morphs of *O. arachnitiformis*. Neither presence/absence nor the colour of the perianth (green versus white) influence attractiveness of the flowers to *Colletes cunicularius* males, the main pollinator of *O. arachnitiformis*.

• **Conclusion** Chemical signals alone can mediate the interactions in highly specialized mimicry systems. Floral colour polymorphism in *O. arachnitiformis* is not subjected to selection imposed by *C. cunicularius* males, and an interplay between different non-adaptive processes may be responsible for the maintenance of floral colour polymorphism both within and among populations.

Key words: *Colletes cunicularius*, floral odour, floral colour polymorphism, mimicry, *Ophrys arachnitiformis*, pollination by sexual deception, pollinator attraction.

INTRODUCTION

It is often assumed that many flowering plants owe much of their floral polymorphism in colour, shape and odour to the combined effects of attracting different pollinator species and to the spatio-temporal variation in the selection regimes they are subjected to (Galen and Kevan, 1980; Pettersson, 1991; Eckhart, 1992; Ollerton *et al.*, 2006). If these determinants of floral polymorphism hold for most cases where the pollinators obtain a reward from their pollination visits, the selection pressures at play may somewhat differ for plants that benefit from pollinator services without offering a reward of any kind to their pollen vectors. Such cases of ‘deceptive’ pollination have evolved multiple times within the angiosperms (Renner, 2006) and they are particularly well represented in the orchid family, where approximately one-third of all 30 000 species known to science bear only rewardless flowers (Dafni, 1984; Nilsson, 1992; Cozzolino and Widmer, 2005; Schiestl, 2005; Tremblay *et al.*, 2005; Jersakova *et al.*, 2006, 2009). Recent studies have reported that pollinators visiting deceptive orchids are likely to leave each inflorescence rapidly visited as no reward is available (Smithson and Macnair, 1996; Smithson and Gigord, 2003). Hence, several authors have suggested that rare morphs within populations could experience higher visitation rates

than more common coexisting morphs, a process known as negative frequency-dependent selection (nFDS; Gigord *et al.*, 2001). This might ultimately promote the persistence of phenotypic variance in floral traits involved in pollinator attraction (Smithson and Macnair, 1997; Ferdy *et al.*, 1998; Gigord *et al.*, 2001). To date, very few studies have combined the quantitative measurement of different floral traits and examined how variation in these traits may be linked to the preferences and learning of the pollinators. Among these studies, high levels of floral odour variation have been found in several deceptive orchids, leading to the suggestion that floral odour polymorphism was probably the result of the absence of selection rather than nFDS mediated by the pollinators (Ackerman *et al.*, 1997; Aragon and Ackerman, 2004; Salzmann *et al.*, 2007). Evolutionary forces other than nFDS may therefore be important in shaping the floral traits of polymorphic orchids.

Among deceptive orchids, those species that achieve cross-pollination by mimicking the mating signals of female insects stand out with their intimate relationships with only one or few pollinator taxa (Kullenberg, 1961; Dafni, 1984; Nilsson, 1992). The specificity in this orchid–pollinator system is based primarily on the mimicry of mating signals of female insects, especially the sex pheromones, by the flower as pollinator attractant (reviewed by Schiestl, 2005; Ayasse, 2006; Vereecken and Schiestl, 2008). Pollination in these orchids is

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FIG. 1. Perianth colour morphs of *Ophrys arachnitiformis* in the study area in southern France showing flowers with a green vs. a white perianth (photographs by N. J. Vereecken).

effected as the male of the targeted insect species attempts copulation with the female decoy of the flower, a phenomenon known as pseudocopulation (Kullenberg, 1961). Several sexually deceptive orchids show discrete intraspecific polymorphism in floral traits, yet contrary to their related food-deceptive counterparts, a narrow range of insect taxa, sometimes perhaps only a single species, ensures pollination (Schiestl, 2005; Schiestl and Schlüter, 2009; Vereecken, 2009). Although a recent study on *Ophrys heldreichii* has reported on the synergistic effect of scent and floral perianth colour in pollinator attraction (Spaethe *et al.*, 2007), the extent to which these results may be applicable to other species within the morphologically diverse genus *Ophrys* remains unknown.

The present study investigated the adaptive significance of perianth colour polymorphism and its influence on pollinator visitation rates in the sexually deceptive orchid *Ophrys arachnitiformis*, a taxon for which the perianth colour is either completely green or translucent (white; Fig. 1). A combination of chemical analyses of floral odours by gas chromatography and mass spectrometry (GC, GC-MS) along with behavioural bioassays performed *in situ* was employed in an experimental design to dissect the floral phenotype and address two major questions. (1) Is floral colour polymorphism associated with differences in floral scent, especially in the patterns of behaviourally active compounds? (2) To what extent are visual signals of the flowers, compared with floral odour, involved in pollinator attraction?

MATERIALS AND METHODS

Study species

The study species, *Ophrys arachnitiformis* Gren. & M. Philippe, grows in calcareous grasslands, garrigues and open woodlands. Its flowering period extends from mid-February until early April and its geographical range extends along the Mediterranean coast from north-eastern Spain to northern Italy (Delforge, 2005). It is primarily pollinated through pseudocopulation by patrolling males of the plasterer bee *Colletes cunicularius* (L.) (Hymenoptera, Colletidae). This orchid species is often found in isolated populations, but it can occasionally be found in sympatry with *O. lupercalis*, one of the few other *Ophrys* species that flowers unusually early in the year. *O. arachnitiformis* was

particularly suited for this study as a considerable body of knowledge has accumulated over the past few years on its floral scent and pollinator behaviour (Peakall and Schiestl, 2004; Mant *et al.*, 2005a; Vereecken *et al.*, 2007; Vereecken and Schiestl, 2008). Furthermore, translocation experiments of plants in pots and the monitoring of individual plants grown in different environments have shown that each colour morph is stable, suggesting that the polymorphism observed has a genetic basis. The frequency of each morph varies among populations, ranging from 100 % of green morphs in some sites of the Rhône river basin to almost 100 % of white morphs in some areas of south-east France and north-east Spain (N. J. Vereecken, pers. obs.).

Sample collection

The floral odour of each colour morph [green (g) vs. white (w)] of *O. arachnitiformis* was sampled in two populations in southern France [Montbazin: $n(g) = 10$, $n(w) = 10$; Montferrier-sur-lez: $n(g) = 12$, $n(w) = 13$]. The two orchid populations investigated for their floral odour were located approx. 20 km apart. Epicuticular waxes of orchids were sampled by extracting fresh and unpollinated flower labella in 200 μ L *n*-hexane (HPLC grade) for 1 min. All extracts were then stored at -20°C .

Chemical analyses

All samples were analysed by GC on an Agilent 6890N chromatograph equipped with a HP-5 capillary column (30 m \times 0.32 mm \times 0.25 μ m). The injector temperature was kept at 300°C . Prior to GC analysis, 100 ng *n*-octadecane was added as internal standard to all samples. Aliquots of the extracts (1 μ L) were injected splitless at 50°C (1 min), followed by a programmed increase of oven temperature to 300°C at a rate of $10^{\circ}\text{C min}^{-1}$; helium was used as the carrier gas. Compounds were identified by comparison of retention times with those of authentic standard compounds. Additionally, selected samples were analysed with a gas chromatograph with a mass-selective detector (Hewlett Packard G1800 A), and MS spectra were compared with those of known reference substances (Mant *et al.*, 2005a). The absolute amounts of the 40 identified compounds were calculated by the

internal standard method as described by Mant *et al.* (2005a). Relative proportions (%) were calculated by summing the absolute amounts of all compounds; absolute amounts of individual compounds were then divided by the total and multiplied by 100.

Behavioural experiments

All behavioural experiments were performed in late March and early April, 2006 in a natural population of the solitary bee *C. cunicularius*, the pollinator of *O. arachnitiformis*, in Cadillon, southern France. All bioassays were conducted between 1000 and 1500 h, when the patrolling activity of *C. cunicularius* males was at its peak. At the study site, hundreds of male bees were patrolling for emerging females in a restricted area. As the males of *C. cunicularius* usually patrol fairly localized regions on the nesting/emergence site (Peakall and Schiestl, 2004), test spots were alternated after each behavioural bioassay to test the responses of different males to the odour blends. Behavioural responses of male bees towards dummies (black cylindrical plastic beads, 4 × 5 mm, mounted on an insect pin) scented with natural extracts of orchid flowers were taped using a voice recorder during a 3-min period and classified into one of two categories: (1) number of approaches [hovering in front of the dummy at close range (<10 cm) without any contact with the odour source], and (2) number of contacts with the scented dummy. Odour sources were presented individually for each test (i.e. each scented dummy was used only once). For each behavioural bioassay, half of each natural extract, representing 100 µL, was applied on each dummy using a 100-µL Hamilton glass syringe. Controls consisted of dummies made odourless by washing them with solvent (HPLC-grade hexane): each was tested independently for their attractiveness after every fifth test by placing them in a male patrolling area after the solvent had evaporated.

To assess the relative role of visual versus odour signals in pollinator attraction, a series of behavioural bioassays were performed by using (1) labellum extracts and (2) floral perianths of plants sampled in Montferrier-en-lez (F). The latter consisted of individual flowers of each colour morph (white vs. green) deprived of their labellum and made odourless by washing them in hexane (HPLC grade). When the visual cues were assayed alone or combined with odour cues, the dummy was fixed at the exactly same location of the flower labellum before it was excised. Individual dummies (controls) were paired with a successive association of (1) a green perianth (GP), (2) a white perianth (WP), (3) a floral odour extract (FE), (4) GP plus FE, and (5) WP plus FE.

Statistical analyses

Means and standard errors of the means (s.e.m.) of absolute (mg mL⁻¹) and relative (%) amounts of all identified compounds were calculated for all natural extracts. A multivariate analysis of floral odour variance in behaviourally active compounds [relative amounts (%)] of *O. arachnitiformis* flowers was performed via a principal components analysis (PCA). A Kruskal–Wallis test and Mann–Whitney *U*-tests were used to compare the total amounts of behaviourally active

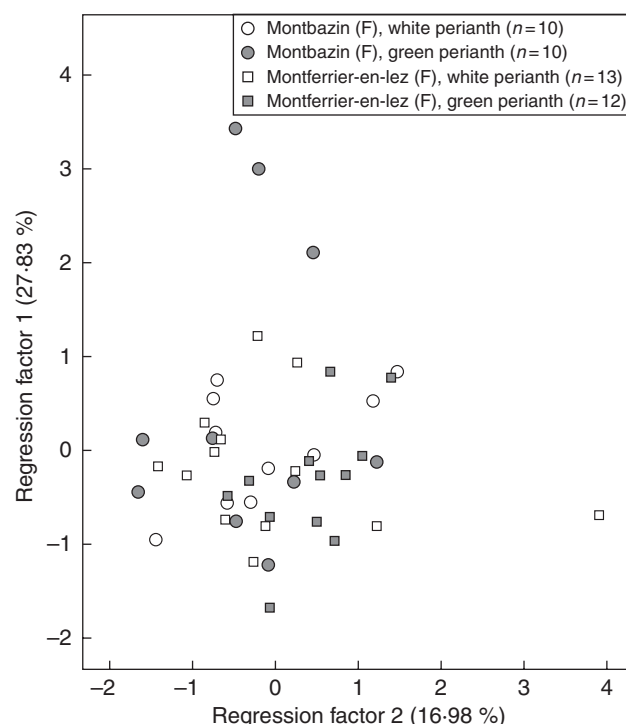


FIG. 2. Floral odour differentiation among colour morphs in *Ophrys arachnitiformis*. Principal component analysis (PCA) of all behaviourally active compounds (relative amounts, %) identified in the pollinator, the solitary bee *Colletes cunicularius*. Regression factors 1 and 2 obtained from the PCA are plotted and account, respectively, for 27.8 and 16.9 % of the variance in proportions of behaviourally active compounds among floral morphs and study sites.

compounds emitted by individual flowers between colour morphs, both within and among populations. To test for differences in male bee responses to natural extracts of orchid flowers, a Kruskal–Wallis test followed by pairwise comparisons of mean values with Mann–Whitney *U*-tests were used. All statistical tests were performed with SPSS 13.0 software with the level of significance set at $\alpha = 0.05$ (Brosius, 2002).

RESULTS

Floral scent differentiation among colour morphs and populations

The odour extracts of fresh, unpollinated flowers of *O. arachnitiformis* consisted mainly of mixtures of long, straight-chained alkanes ranging from 21 to 29 carbon atoms and their associated alkenes, which have previously been shown to trigger mating behaviour in patrolling *C. cunicularius* males (Mant *et al.*, 2005a). With only 44.8 % of the total floral odour variance explained among samples, colour morphs and populations, multivariate analysis failed to detect any clustering according to colour morph in *O. arachnitiformis* (Fig. 2). In particular, examination of the patterns (%) of (Z)-7 alkenes with 21, 23 and 25 carbon chain length identified by Mant *et al.* (2005a) as key attractants for *C. cunicularius* did not differ significantly between morphs within populations (ANOVA, $F_{3,41}$, all $P > 0.05$). These results indicate that differences in perianth colour (green versus white) are not associated with detectable differences in odour, either in relative amounts (Fig. 2) or in absolute

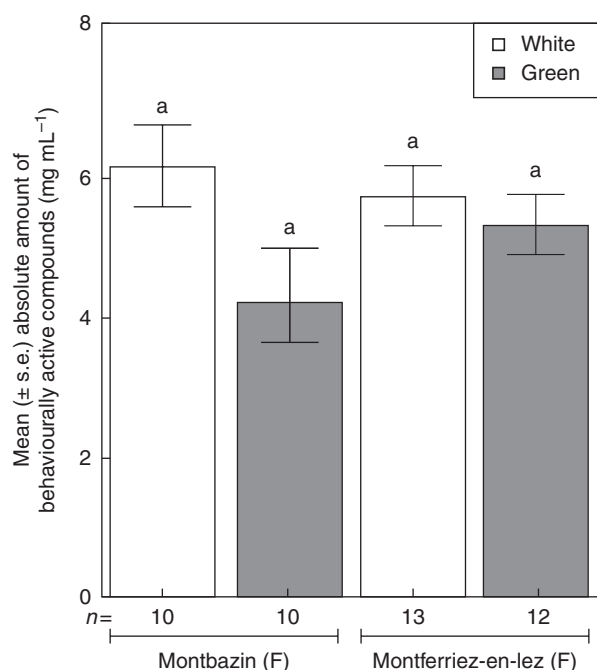


FIG. 3. Floral odour differentiation among colour morphs both within and among populations in absolute amounts of all behaviourally active compounds identified in the pollinator, the solitary bee *Colletes cunicularius*. Kruskal–Wallis test and Mann–Whitney *U*-tests, $\alpha = 0.05$. Identical letters above error bars indicate the absence of significant differences; the number of samples analysed is listed beneath the columns.

amounts of behaviourally active compounds produced by the labellum within populations (Fig. 3; Mann–Whitney *U*-tests: Montbazin $P = 0.105$; Montferriez $P = 0.503$). In addition, the flowers sampled in two different populations emitted similar absolute amounts of behaviourally active compounds (Fig. 3; Kruskal–Wallis test: $\chi^2_3 = 3.694$, $P = 0.296$).

Floral signals and pollinator attraction

Floral scent was the only floral trait capable of triggering significant levels of pollinator attraction compared with controls (plastic beads washed in hexane) (Fig. 4; Kruskal–Wallis test χ^2_5 approaches = 89.902, $P < 0.0001$; χ^2_5 contacts = 93.975, $P < 0.0001$). The addition of a coloured perianth to the controls or to the scent source did not lead to an increase in visitation rates by the male bees (Fig. 4; control vs. coloured perianth: Kruskal–Wallis test χ^2_2 approaches = 2.227, $P = 0.328$; χ^2_2 contacts = 0.105, $P = 0.949$; scented dummy vs. scent and coloured perianth: Kruskal–Wallis test χ^2_2 approaches = 0.188, $P = 0.910$; χ^2_2 contacts = 3.394, $P = 0.183$), which suggests that pairing a visual signal to the odour source had no effect on the attractiveness towards male bees.

DISCUSSION

Floral scent differentiation among floral morphs and populations

In the present study, there was no evidence for floral odour differentiation in either relative or absolute amounts between colour morphs in the sexually deceptive orchid *Ophrys arachnitiformis*

(Figs 2 and 3). Similar results have been reported in other plant taxa such as *Corydalis cava* (Fumariaceae; Olesen and Knudsen, 1994), *Hesperis matronalis* (Brassicaceae; Majetic *et al.*, 2008) and *Polemonium viscosum* (Phlox family, Polemoniaceae; Galen and Kevan, 1980; Galen, 1985), although in *P. viscosum* correlations were found between scent morphs and other parameters such as the intraspecific variation in flower and inflorescence morphology (Galen *et al.*, 1987). By contrast, Salzmann and Schiestl (2007) found that flowers of different colours of the food-deceptive orchid *Dactylorhiza romana* emitted identical mean proportions of odour compounds, with the exception of linalool (3,7-dimethylocta-1,6-dien-3-ol), which differentiated yellow versus orange and red morphs in this orchid. Likewise, Pecetti and Tava (2000) found a significant differentiation in floral scent among colour morphs of *Medicago sativa* (alfalfa, Fabaceae), with a trend for dark flowers to emit larger amounts of floral volatiles than light-coloured morphs. Odell *et al.* (1999) also showed that floral colour polymorphism in *Antirrhinum* spp. (Scrophulariaceae) is paralleled by qualitative differences in floral scent emission, yellow flowers (the wild type) releasing methyl benzoate whereas white-flowered (domesticated) strains produce methyl cinnamate. And finally, Büsser (2004) found evidence for phenotypic correlation between variation in floral colour and scent in *Mimulus aurantiacus* (Scrophulariaceae), a species characterized by three morphs, each associated with a different pollination syndrome. Despite these recent advances in understanding how variation in some floral traits might be linked to variation in other traits, none of these studies has examined whether intra-specific floral differences are perceived by the pollinators and how such differences might ultimately translate into differential visitation rates (Kunze and Gumbert, 2001; Salzmann and Schiestl, 2007), especially in generalized pollination systems (see discussion in Herrera *et al.*, 2006).

Although several studies have reported on floral odour differentiation among populations (Knudsen, 2002, and references therein; Dötterl *et al.*, 2005; Svensson *et al.*, 2005; Salzmann *et al.*, 2007; Majetic *et al.*, 2008), the present analyses failed to provide evidence of floral odour differentiation among populations in *O. arachnitiformis* (Fig. 2). These results might at first glance seem counterintuitive, given that Mant *et al.* (2005b) reported on population-specific floral odour signals in *O. exaltata*, which is also pollinated by *C. cunicularius* males. We suggest that the absence of population differentiation in floral odour signals in the present study is due to the geographical proximity (distance 20 km) of the populations investigated. This hypothesis is supported by a similar effect of geographical distance on the differentiation of the female sex pheromone signal in *C. cunicularius*, the model species, for which neighbouring populations separated by distances of less than 40 km (N. J. Vereecken *et al.*, unpubl. res.) emit virtually identical blends of sex pheromone compounds, although population-specific ‘dialects’ were detected on a larger geographical scale across western Europe (Vereecken *et al.*, 2007).

Odour vs. colour signals in *Ophrys* pollination

The present bioassays show that *Ophrys* floral odour constitutes the major pollinator attractant in the orchid pollinator

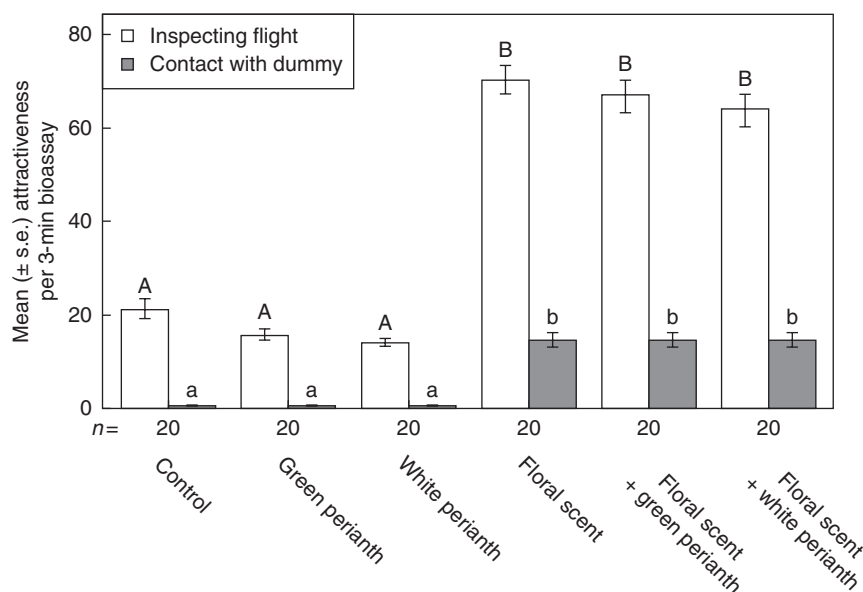


FIG. 4. An assessment of the relative role of visual vs. odour cues in pollinator attraction in *Ophrys arachnitiformis*. Visual cues (green versus white perianths) and odour cues (floral odour extract) were assayed both individually and in combination for their attractiveness to patrolling males of *Colletes cunicularius*. Kruskal–Wallis test and Mann–Whitney *U*-tests, $\alpha = 0.05$. Different letters above error bars indicate significant differences in attractiveness; the number of replicates is listed beneath the columns.

species pair investigated (see also Schiestl *et al.*, 1999). Neither the presence of a perianth nor its colour influenced visitation rates of scented dummies by patrolling males of *C. cunicularius* (Fig. 4). We therefore suggest that the ‘search image’ (*sensu* Tinbergen, 1960; Gegear and Laverty, 2001) of a mating partner (i.e. a female insect or an *Ophrys* flower) by *C. cunicularius* males is based primarily on odour signals, namely proportional patterns of a specific set of behaviourally active compounds (see also Vereecken and Schiestl, 2008). The results contrast markedly with those of a recent study by Spaethe *et al.* (2007), who reported a significant increase in pollinator attraction through an interactive and synergistic effect between olfactory cues produced by the labellum on the one hand and visual signals (a large pinkish perianth) on the other in *Ophrys heldreichii* from Crete. The authors proposed that selection may have favoured the spectral resemblance between the pinkish perianth of the flowers of *O. heldreichii* and the overall reflectance of the host plants visited by the females of the pollinator, *Tetralonia berlandi*, during their foraging bouts.

The two solitary bee species investigated here and by Spaethe *et al.* (2007) have contrasting ecologies and reproductive strategies. The females of *C. cunicularius* have largely generalized floral choices (Müller and Kuhlmann, 2008) and tend to form large, dense and compact colonies on bare, sandy soil sometimes covered by low vegetation, where mating usually takes place as soon as the females emerge from their underground brood cell. Conversely, the females of *T. berlandi* visit a restricted spectrum of host plants for pollen, including some species of Fabaceae, and their nests tend to be scattered across their preferred habitat. During the reproductive season, males of *T. berlandi* therefore patrol the preferred forage plants of the females and initiate mating at these ‘rendezvous’ flowers (Alcock *et al.*, 1978; Westrich,

1989; Paxton, 2005), which have an overall similar spectral reflectance to the perianth of *O. heldreichii* (Spaethe *et al.*, 2007). These two contrasting case studies therefore suggest that (1) wild bees can differ greatly in their reproductive strategies, (2) these differences can have an important impact on the sensory ecology of the species and on the signals involved in mate finding and recognition, and (3) generalization of the importance of one signal type over another should be made with great caution in this group of orchid pollinators. Several studies have reported that mate location by patrolling males in *C. cunicularius* is mediated by the sex pheromone released by virgin, emerging females (Cane and Tengö, 1981; Borg-Karlson *et al.*, 2003; Mant *et al.*, 2005a), and the present findings suggest that chemical signals might be the prevalent communication channel used by patrolling *C. cunicularius* males in search of females. In summary, the present results illustrate that visual cues displayed by flowers of *O. arachnitiformis* play virtually no role in pollinator attraction. To summarize, the results show that perianth colour has no effect on pollinator attraction (male approaches per time) in the behavioural context tested in this study. However, the results cannot exclude the possibility that perianth colour plays a role in other aspects of pollinator attraction that were not explicitly tested, for instance in the detection of flowers and subsequent learning and avoidance of deceptive plants after unsuccessful copulation attempts by male bees (see also Streinzer *et al.*, 2009).

Finally, the results suggest that because pollinator attraction is primarily odour-mediated, and ‘neutral’ morphological overlap can occur between species, caution should be taken when species identification is based only on morphological traits in *Ophrys*. The two floral morphs investigated here flower at the same period of the year, are often found in sympatry in population-specific proportions, and they both attract

males of *C. cunicularius* on a highly species-specific basis. Yet, there is no consensus in the contemporary literature on whether these colour morphs should be considered as different species (e.g. Bournérias and Prat, 2005) or as varieties (e.g. Delforge, 2005). The present results clearly indicate that the colour of the perianth had no effect on the visitation rates of patrolling males of *C. cunicularius* to individual flowers (Fig. 4), suggesting that these colour morphs do not merit taxonomic recognition of any kind.

Maintenance of floral colour polymorphism in *Ophrys*

The present study investigated the adaptive role of floral colour polymorphism in *Ophrys*. The results of chemical analyses (Figs 2 and 3) and bioassays (Fig. 4) suggest that floral colour polymorphism in *O. arachnitiformis* is apparently not subjected to selection imposed by *C. cunicularius* males, its main pollinator species across the whole of its geographical range. Therefore, we suggest that a series of adaptive processes such as (1) spatially and temporally variable selection pressures imposed by different pollinators (see reviews by Herrera, 1996; Waser *et al.*, 1996; Cresswell, 1998; Johnson and Steiner, 2000; Aigner, 2006; Herrera *et al.*, 2006), (2) negative frequency-dependent selection (e.g. Smithson and Macnair, 1997; Gigord *et al.*, 2001) and (3) disruptive selection (Smithson and Macnair, 1996) are presumably not responsible for the maintenance of the floral polymorphism observed in *O. arachnitiformis*.

We hypothesize that, contrary to floral odour variation, which is under pollinator-mediated selection in *Ophrys* (Mant *et al.*, 2005b; Vereecken and Schiestl, 2008; Cortis *et al.*, 2009), floral colour polymorphism both within and among populations in *O. arachnitiformis* is more likely to be the result of non-adaptive processes, perhaps along with non-pollinator agents of selection such as herbivores, florivores or floral parasites (e.g. Strauss and Whittall, 2006; Theis, 2006; Nuismer and Ridenhour, 2008). A study of population genetic structure using microsatellite markers conducted by Mant *et al.* (2005b) on *O. exaltata* (pollinated by *C. cunicularius* males) has yielded low F_{ST} values (range 0.005–0.127, mean 0.075), indicating large effective population sizes and suggesting that gene flow among populations might be important in this orchid species. Gene flow can be an important evolutionary force in the maintenance of colour polymorphism, even in conjunction with random genetic drift (Gray and McKinnon, 2006), and it has been advocated as an important component of the evolution of orchids (e.g. Aragon and Ackerman, 2004; Tremblay *et al.*, 2005), particularly in the Mediterranean region with its human-altered, fragmented habitats and populations (Thompson, 2005). Gene flow among populations in flowering plants can typically occur via seeds or via pollen, yet while some pollinators are capable of mediating long-distance pollen dispersal (Janzen, 1971; Peakall and Beattie, 1996), Peakall and Schiestl (2004) demonstrated that males of *C. cunicularius* patrol only a fairly restricted region of their reproductive site looking for potential mates, typically less than 100 m in circumference. Their limited flight routes therefore constrain long-distance pollen movement, and gene flow among orchid populations, when it occurs, is probably the outcome of a long-range

dispersal of the typical dust-like orchid seeds. This hypothesis requires confirmation from parallel studies to evaluate specifically the relative importance of pollen versus seed dispersal in *Ophrys* species.

CONCLUDING REMARKS

Despite the reduction in the array of potential floral visitors/pollinators due to the filtering effect of combining different floral signals, plant–pollinator interactions are still largely generalized. This situation makes investigations of functional floral ecology considerably more complicated, particularly when conducting experimental studies on the relative role of floral functional traits or on the adaptive significance of trait polymorphism at different spatial, temporal and taxonomic scales. Floral traits are commonly interdependent and combine to varying degrees to determine pollinator attraction and fidelity (Roy and Raguso, 1997; Chittka *et al.*, 1999; Raguso and Willis, 2002, 2005; Kunze and Gumbert, 2001; Goyret *et al.*, 2007, and references therein; but see Odell *et al.*, 1999). Yet, as pointed out by Fenster *et al.* (2004) and Raguso (2006), there is a dramatic lack of information on how floral traits interact, how they attract the pollen vector(s), and what drives their changes in composition over evolutionary time. Here, we were able to shed light on some of these important issues by showing that floral scent is the key to pollinator attraction in one sexually deceptive orchid and that visual signals have no effect on pollinator attraction in this highly specialized mimicry system. We are confident that future studies integrating analyses of floral scents, pollinator behaviour and sensory ecology will provide considerable insight into the evolutionary forces that shape the signals mediating insect/pollinator interactions, particularly in specialized pollination systems (see also Raguso, 2008a, b; Schiestl and Schlüter, 2009).

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